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Studies on the Isolation of *Listeria monocytogenes* from Food, Water, and Animal Droppings: Environmental Health Perspective

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1. Introduction

Veterinarians, Medical doctors, Environmentalists and other scholars know listeriosis by various names such as tiger river disease, silage sickness, leukocytosis, cheese sickness. Interestingly, Gustav Hülphers first discovered the bacteria and named it *Bacillus hepatis*. However, he did not preserve his bacterial strains, and the bacterium was later recognized as *Listeria monocytogenes* (Vishal. 2004). Fifteen years later, Murray *et al.* (1926) also identified bacteria identical to *L. monocytogenes*, as the cause of monocytosis in rabbit and guinea pig. The scholars preserved the isolates of the bacteria (ATCC no. 15313; ATCC no. 4428) so the credit goes to Murray *et al.* for isolation of *L. monocytogenes* for the first time. In addition to the existing knowledge, Pirie finally named the species *L. monocytogenes* in 1940 and thereafter it was included in the 6th edition of Bergey's Manual of Determinative Bacteriology (1948).

In the early 1980's Listeriosis was classified under anthroozoonoses, which was changed to amphixenoses in the late 1990s. It lacks its true definition of Zoonotic disease because of involvement of an inanimate reservoir (food and Environment) as the major cause of listeriosis. Up to 1961, *L. monocytogenes* was regarded as the one and only species of the genus *Listeria* but later other species have been identified. Listeriosis is of great public health concern because of its high mortality (20 to 30%) and its common source epidemic potential. The most important aspect of this organism in food hygiene is the ability of the bacteria to survive in a wide range of temperatures and to make biofilms on various environmental surfaces, which serve as natural habitats or reservoirs (Duggan and Phillips, 1998). Direct transmission is possible, especially among veterinarians, performing gynecological interventions with aborted animals. Animals may be diseased or asymptomatic carriers of *L. monocytogenes* shedding the organism in their faeces. Thus, earlier it was believed that *L. monocytogenes* was causing disease by direct transmission from animals to humans. Today it is generally considered that ingestion is the main mode of infection with food and water being the main vehicles of infection. A listeriosis outbreak in the Maritime Provinces of Canada (1981) was indeed related to food but

it was not until the outbreak of California from January to August 1985 (Linnan *et al.*, 1988) that food was recognized as an important vehicle of *Listeria* transmission. According to Mead *et al.* (1999) food is an important vehicle of *Listeria* transmission in 99% of listeriosis cases. Risk assessments by the World Health Organisation (WHO) have estimated that 99% of all Listeriosis could be eliminated if the *L. monocytogenes* level never exceed 1000 cfu/g in food at the point of consumption. Nosocomial infection has also been described, placing physicians and other medical staff at risk.

Listeria monocytogenes is a Gram-positive rod-shaped bacterium. It is the agent of **listeriosis**, a serious infection caused by eating food contaminated with the bacteria. Listeriosis has been recognized as an important public health problem in the United States. The disease affects primarily pregnant women, newborns, and adults with weakened immune systems.

Listeriosis is a serious disease for humans; the **overt form** of the disease has mortality greater than 25 percent. The two main clinical manifestations are sepsis and meningitis. Meningitis is often complicated by encephalitis, a pathology that is unusual for bacterial infections.

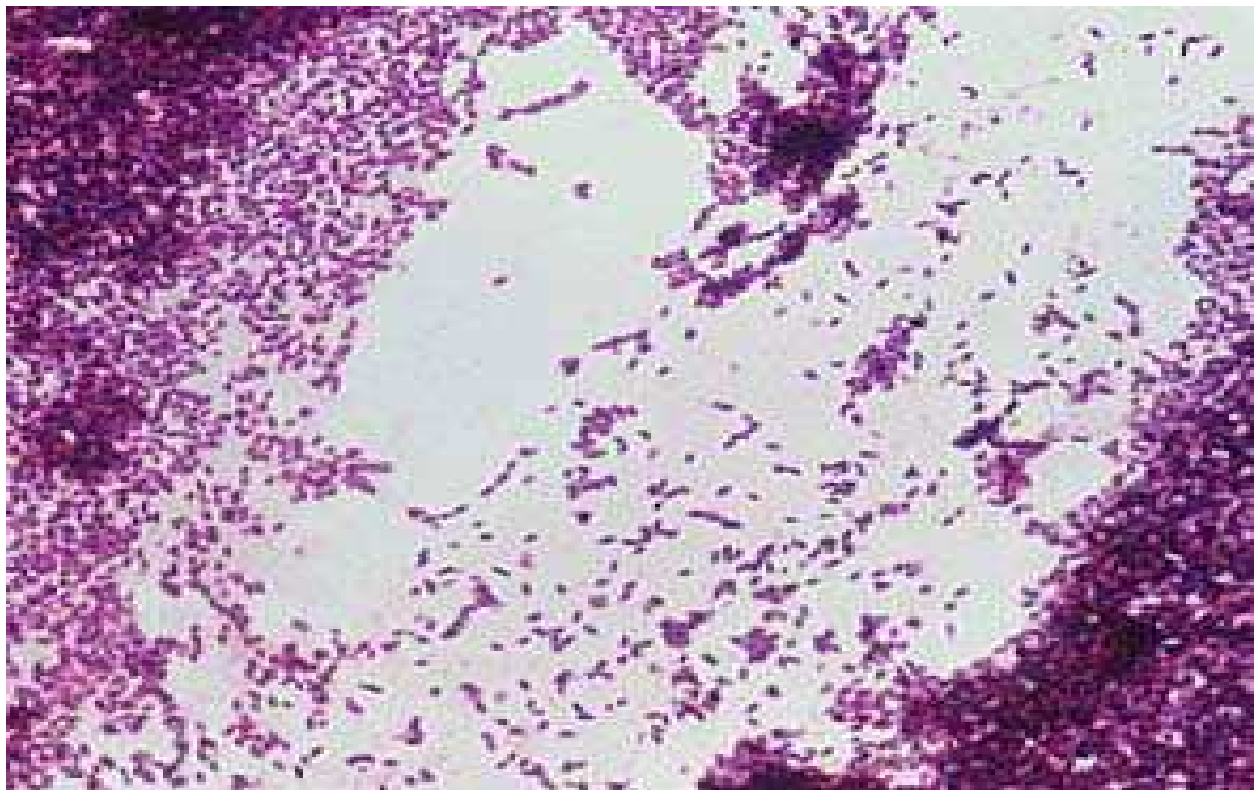


Fig. 1. *Listeria monocytogenes* Gram Stain as seen in a light Microscope (Kenneth, 2004).

Microscopically, *Listeria* species appear as small, Gram-positive rods, which are sometimes arranged in short chains. In direct smears they may be coccoid, so they can be mistaken for streptococci. Longer cells may resemble *Corynebacteria*. Flagella are produced at room temperature but not at 37°C. Hemolytic activity on blood agar has been used as a marker to distinguish *Listeria monocytogenes* among other *Listeria* species, but it is not an absolutely definitive criterion. Further biochemical characterization may be necessary to distinguish between the different *Listeria* species (Linnan *et al.*, 1988).

As Gram-positive, non-spore forming, catalase-positive rods, the genus *Listeria* was classified in the family Corynebacteriaceae through the seventh edition of Bergey's Manual. 16S rRNA cataloging studies of Stackebrandt et al. (1983) demonstrated that *Listeria monocytogenes* was a distinct taxon within the *Lactobacillus-Bacillus* branch of the bacterial phylogeny constructed by Woese (1981). In 2001, the Family **Listeriaceae** was created within the expanding Order **Bacillales**, which also includes Staphylococcaceae, Bacillaceae and others. Within this phylogeny there are six species of *Listeria*. The only other genus in the family is *Brochothrix*.

2. Natural habitats of *Listeria* and incidence of disease

Until about 1960, *Listeria monocytogenes* was thought to be associated almost exclusively with infections in animals, and less frequently in humans. However, in subsequent years, listeriae, including the pathogenic species *L. monocytogenes* and *L. ivanovii*, began to be isolated from a variety of sources, and they are now recognized to be widely distributed in Nature. In addition to humans, at least 42 species of wild and domestic mammals and 17 avian species, including domestic and game fowl, can harbor listeriae. *Listeria monocytogenes* is reportedly carried in the intestinal tract of 5-10% of the human population without any apparent symptoms of disease. Listeriae have also been isolated from crustaceans, fish, oysters, ticks, and flies (Doyle and Schoeni, 1986).

The term **listeriosis** encompasses a wide variety of disease symptoms that are similar in animals and humans. *Listeria monocytogenes* causes listeriosis in animals and humans; *L. ivanovii* causes the disease in animals only, mainly sheep. Encephalitis is the most common form of the disease in ruminant animals. In young animals, visceral or septicemic infections often occur. Intra-uterine infection of the fetus via the placenta frequently results in abortion in sheep and cattle (Doyle and Schoeni, 1986).

The true incidence of listeriosis in humans is not known, because in the average healthy adult, infections are usually asymptomatic, or at most produce a mild influenza-like disease. Clinical features range from mild influenza-like symptoms to meningitis and/or meningoencephalitis. Illness is most likely to occur in pregnant women, neonates, the elderly and immunocompromised individuals, but apparently healthy individuals may also be affected. In the serious (overt) form of the disease, meningitis, frequently accompanied by septicemia, is the most commonly encountered disease manifestation. In pregnant women, however, even though the most usual symptom is a mild influenza-like illness without meningitis, infection of the fetus is extremely common and can lead to abortion, stillbirth, or delivery of an acutely ill infant (David et al., 1994).

In humans, overt listeriosis following infection with *L. monocytogenes* is usually sporadic, but outbreaks of epidemic proportions have occurred. In 1981, there was an outbreak that involved over 100 people in Canada. Thirty-four of the infections occurred in pregnant women, among whom there were nine stillbirths, 23 infants born infected, and two live healthy births. Among 77 non pregnant adults who developed overt disease, there was nearly 30% mortality. The source of the outbreak was coleslaw produced by a local manufacturer (David et al., 1994.)

In 1985, in California, 142 people developed overt listeriosis. Of these, 93 cases were perinatal, and among the 49 cases that were in non pregnant individuals, 48 were

immunocompromised. Thirty fetuses or newborn infants died and 18 adults died. The source of the bacteria was a certain brand of "pasteurized" soft cheese that apparently got contaminated with non pasteurized (raw) milk during the manufacturing process (Kenneth, 2004)..

In 2002, a multistate outbreak of *Listeria monocytogenes* infections with 46 culture-confirmed cases, seven deaths, and three stillbirths or miscarriages in eight states was linked to eating sliced turkey deli meat. One intact food product and 25 environmental samples from a poultry processing plant yielded *L. monocytogenes*. Two environmental isolates from floor drains were indistinguishable from that of outbreak patient isolates, suggesting that the plant might be the source of the outbreak (Kenneth, 2004).

Listeria monocytogenes, commonly referred to as *Listeria*, is a pathogen that causes listeriosis, a serious human illness. It is unlike most other foodborne pathogens because it can grow at proper refrigeration temperatures. In addition, *Listeria* is widely distributed in nature, and the organism has been recovered from farm fields, vegetables, animals and other environments such as food processing facilities, retail stores and home kitchens and ready-to-eat foods (Kenneth, 2004).

3. Symptoms and disease process

L. monocytogenes causes listeriosis, a serious infection with high hospitalization rates for those who become ill. People at highest risk for a severe case include the elderly, the fetuses of pregnant women, and the immunosuppressed. It is unique among foodborne pathogens since its incubation time (time from ingestion of cells to illness) is at least seven days. Listeriosis is a rare disease with a high mortality rate, causing about 43 percent of the food poisoning deaths in the United States.

L. monocytogenes can also cause mild, flu-like symptoms in healthy individuals when consumed at very high levels. A person with listeriosis has fever, muscle aches and occasional gastrointestinal symptoms such as nausea or diarrhea. If infection spreads to the nervous system, symptoms such as headache, stiff neck, confusion, loss of balance, or convulsions can occur. Infected pregnant women may experience only a mild, flu-like illness; however, infections during pregnancy can lead to miscarriage or stillbirth, premature delivery or infection of the newborn (Vishal, 2004).

Primary routes of transmission

Foods can become contaminated with *L. monocytogenes* along the continuum from farm to fork, in the produce growing environment, during processing, or during handling and preparation in retail establishments and consumers' kitchens (ILSI, 2005).

The primary route of transmission is through the ingestion of contaminated food. The International Life Sciences Institute in 2005 described high-risk foods for causing listeriosis as those with the following properties:

1. have the potential for contamination with *L. monocytogenes*;
2. support the growth of *L. monocytogenes* to high numbers;
3. are ready-to-eat;
4. require refrigeration; and
5. are stored for an extended period of time(ILSI, 2005).

Because *Listeria* is abundant in nature and can be found almost anywhere, there can be a constant re-introduction of the organism into the food plant, retail setting, foodservice establishment and home. It is difficult to totally eliminate this contaminant from the food-handling environment, but the goal is to control it as effectively as possible, especially where it can contaminate ready-to-eat, refrigerated foods.

Although *L. monocytogenes* is the only member of the *Listeria* family that causes human illness, the presence of any member of the *Listeria* family in a food processing environment may indicate that conditions are favorable for *L.monocytogenes* proliferation(ILSI,2005)..

4. Control

Effective control of *L. monocytogenes* requires prevention of contamination (to the extent possible) and prevention of growth through time/temperature or formulation control. Knowledge of potential harborage sites is important, as contamination is more likely to occur when the organism has become established in a niche. Food processing plant surveys have found *Listeria* in the following locations (listed approximately in the order of prevalence):

- floors
- drains
- coolers
- cleaning aids such as brushes, sponges, etc.
- product and/or equipment wash areas
- food contact surfaces
- condensate
- walls and ceilings

4.1 Compressed air (ILSI, 2005)

Control of *Listeria* relies on detecting and managing harborage sites with thorough and frequent cleaning. This includes daily cleaning of floors and drains, and adequate attention to less frequently cleaned areas such as HVAC systems, walls, coolers and freezers. Also, damaged equipment, cracks, crevices and hollow areas must be part of sanitation and inspection schedules. It is essential to avoid creation of aerosols during cleaning, especially of floors and drains, to avoid spread of contaminants.

The organism is killed by normal food pasteurization and cooking processes, and is typically sensitive to most sanitizers at recommended rates. Contamination may occur after the cooking process in the processing environment, at retail locations and in the home. For example, post pasteurization contamination of food products can occur when the organism is dispersed via an aerosol. Prevention of growth is essential to avoid the potential for illness, because *L. monocytogenes* can grow at refrigerated temperatures, defeating one of the traditional food safety measures.

L. monocytogenes can survive on cold surfaces and can also multiply *slowly* at 34° F. It has also been shown to grow to a water activity as low as 0.92 and over a pH range of 4.4-9.4(ICMSF, 2004). Because the organism can grow under refrigeration, effective labeling to ensure product rotation in retail settings is an important control measure for ready-to-eat products.

Since this organism continues to elicit concern among consumers, regulators, processors and retailers, studies need to be carefully designed to ensure validity (ILSI, 2005).

5. Background on challenges to the zero tolerance initiative

The FDA/Food Safety and Inspection Service risk assessment reinforces epidemiological conclusions that food borne Listeriosis is a moderately rare, although severe, disease. A study by the Food Products Association showed it is likely that low levels of *L. monocytogenes* are consumed routinely with limited effect. It is believed that 5 percent of the general population may be asymptomatic carriers of *Listeria*, but the percentage may be higher in particular groups, such as slaughterhouse workers.

Extensive risk assessments and analyses have been conducted by the Food Safety and Inspection Service (FSIS, 2005), U.S. Food and Drug Administration/FSIS, World Health Organization/Food and Agriculture Organization and International Life Sciences Institute to identify factors that contribute to risk of illness (Gombas *et al.*, 2003). This research is important because the prevalence in the food supply does not match the rate of illness in the population, and because the outcome of illness in susceptible individuals is very severe. These assessments have generally concluded that the ability of a food to support growth of *Listeria* enhances risk. Because of this, US FDA issued draft Compliance Policy Guidelines in February, 2008 based on the ability of a product to support growth of *L. monocytogenes* (ILSI, 2005). The USDA retains a zero tolerance policy for RTE foods with respect to *L. monocytogenes*, while other countries allow up to 100 Cfu/g in certain foods (ILSI, 2005).

6. *Listeria monocytogenes* in the human and animal environment

Listeriosis is essentially a food borne disease caused by *Listeria monocytogenes* and to some extent *L. ivanovii*. The disease conditions vary from severe invasive forms that affect immunocompromised patients to febrile gastroenteritis and perinatal infections associated with fetal loss or abortion in humans and animals (Siegmán-Igra *et al.*, 2002). Although rare, the disease is reported (Lyautey *et al.*, 2007) to have a very high mortality rate (20-50%), thus making it of serious public health concern. Despite the general consensus that food is the primary route of transmission of this disease, wastewater has long been reported to be a potential reservoir for *Listeria* species and possible source of transmission (Paillard *et al.*, 2005; Arslan and Ozdemir, 2008). Watkins and Sleath (1981) reported the prevalence of *Listeria* species in sewage at numbers far higher than those of *Salmonella* species. And recent studies suggest that *Listeria* species readily survive conventional wastewater treatment processes even after tertiary treatment (Paillard *et al.*, 2005).

With reports of inadequate removal of *Listeria* pathogens from wastewater coming from the developed world (Paillard *et al.*, 2005), one can safely presume that wastewater treatment plants in developing countries such as South Africa are inefficient at removing these pathogens from wastewater influents prior to discharge of the final effluents into the receiving waters for obvious reasons. Most studies (Mackintosh and Colvin, 2003; Obi *et al.*, 2007; Obi *et al.*, 2008) in the area of water quality in South Africa had focused almost exclusively on drinking or potable water supply with scanty reports in the literature on treated wastewater effluent as a source of pathogens for receiving waters. This may have serious public health implications as about 80 % of South Africans are reported to depend

on surface water bodies for drinking, domestic and agricultural purposes (Mackintosh and Colvin, 2003). It is little surprise therefore that about 43, 000 deaths (mostly children) are reported annually in South Africa due to diarrhea diseases (Mara, 2001). The situation is amongst the worst in the Eastern Cape Province due to high level of poverty, low level of sanitation, and lack of appropriate infrastructure (Mackintosh and Colvin, 2003). While reports in the media suggests that cholera may be responsible for the majority of these infections, actual diagnosis suggests that these diseases could have been caused by any other waterborne pathogen apart from *Vibrio* species. A case in point was seen in the report of the Daily Dispatch of Thursday, 30th of January 2003, where out of 446 cases of water related diseases reported to the Eastern Cape health authorities, only 25 (5.6 %) were confirmed to be cholera and yet the disease was termed a “cholera outbreak” without ascertaining the true identities of the pathogens responsible for over 84% of reported cases.

There is a general belief that the larger population of bacteria species grow as adherent to surfaces in all nutrient-sufficient aquatic ecosystems and that these sessile bacterial cells differ profoundly from their planktonic (free-living) counterpart (Costerton *et al.*, 1978). It has also been reported that the existence of pathogens as free-living or plankton-associated cells, is critical to their survival in the environment as well as their transmission from one host to another (Donlan and Costerton, 2002). Several studies have revealed the preponderance of *Listeria* species to exist as biofilms attached to surfaces such as stainless steel, glass and propylene (Mafu *et al.*, 1990), and food and food processing environments (Lunden *et al.*, 2000). There is however little or no report in the literature on *Listerio*-plankton association in the natural environment. Understanding the distribution of *Listeria* cells as free-living or plankton-associated niches may provide clues on how best to reduce the survival potentials of these pathogens in the environment and during wastewater treatment, and consequently reduce their ability to interact with human and animal populations.

Nwachukwu *et al.*, 2010 reported the isolation of *Listeria monocytogenes* from two anthropogenic lakes from Lokpa- Ukwu, Abia State (Nigeria). The identification of isolates was based on cultural and morphological appearances. The identity of isolates was confirmed by biochemical tests. The results of this study revealed that the pathogen was present in 22 out of 24 samples of water from Lake A, giving a prevalence rate of 91.67% while in lake B, the prevalence rate observed was 79.17%. This high prevalence rate is not surprising as the organism is quite ubiquitous in soil, water, and animal dung samples. *Listeria monocytogenes* is water and food borne bacterial pathogen that is ubiquitous in nature and shows ability to persist in its environment for prolong time (Yutaka *et al.*, 2004).

7. *Listeria monocytogenes* in food and food industries

L. monocytogenes is a bacterium that can contaminate foods and cause a mild non-invasive illness (called listerial gastroenteritis) or a severe, sometimes life-threatening, illness (called invasive listeriosis). Persons who have the greatest risk of experiencing listeriosis after consuming foods contaminated with *L. monocytogenes* are fetuses and neonates who are infected after the mother is exposed to *L. monocytogenes* during pregnancy, the elderly, and persons with weakened immune systems. Invasive listeriosis is characterized by a high case-fatality rate, ranging from 20 percent to 30 percent (USFDA, 2003).

L. monocytogenes is widespread in the environment. It is found in soil, water, sewage, and decaying vegetation. It can be readily isolated from humans, domestic animals, raw

Category	Potential Sources of <i>L. monocytogenes</i>
A. Ingredients	Raw foods, such as: Raw meat, poultry, and seafood Raw milk Raw produce
B. Processing aids	Compressed air Ice Brine solutions used in chilling refrigerated RTE foods
C. Contact surfaces for RF-RTE foods	Fibrous and porous-type conveyor belts Filling and packaging equipment Belts, peelers, and collators Containers, bins, tubs and baskets Slicers, dicers, shredders and blenders Utensils Gloves
D. Surfaces that do not contact RF-RTE foods	In-floor weighing equipment Cracked hoses Hollow rollers for conveyances Equipment framework Wet, rusting, or hollow framework Open bearings within equipment Poorly maintained compressed air filters Condensate drip pans Motor housings Maintenance tools (e.g., wrenches and screw drivers) Forklifts, hand trucks, trolleys, and racks On/off switches Vacuum cleaners and floor scrubbers Trash cans and other such ancillary items Tools for cleaning equipment (e.g., brushes and scouring pads) Spiral freezers/blast freezers Ice makers Aprons
E. Plant environment	Floors, walls and drains Ceilings, overhead structures, and catwalks Wash areas (e.g., sinks), condensate, and standing water Wet insulation in walls or around pipes and cooling units Rubber seals around doors, especially in coolers Contents of vacuum cleaners

(SOURCE: Quin et al., 1999; Slustsker and Schuchat, 1999 ; Afnor, 2000 ; EUAHS,2007).

Table 1. Potential Sources of *L. monocytogenes*Scenarios That Could Lead to Contamination with *L. monocytogenes*

agricultural commodities, and food processing environments (particularly cool damp areas) (NACMCF, 1991). Control of *L. monocytogenes* in the food processing environment has been the subject of a number of scientific publications (Fenlon *et al.*, 1996). *L. monocytogenes* can multiply slowly at refrigeration temperatures, thereby challenging an important defense against food-borne pathogens. (Doyle et al, 2001).

Most cases of human listeriosis occur sporadically - that is, in an isolated manner without any apparent pattern. However, much of what is known about the epidemiology of the disease has been derived from outbreak-associated cases, in which there is an abrupt increase in reports of the disease. With rare exceptions, foods that have been reported to be associated with outbreaks or sporadic cases of listeriosis have been foods that can support the growth of *L. monocytogenes* and that are ready-to-eat (including coleslaw, fresh soft cheese made with unpasteurized milk, frankfurters, deli meats, and butter) (USFDA, 2003). Outbreaks of listeriosis are often associated with a processing or production failure (Slutsker and Schuchat 1999); this association has been less evident among sporadic cases (Slutsker and Schuchat 1999).

In addition to this information obtained from reported cases of listeriosis, contamination data (largely obtained from samples of foods collected at retail or during storage before sale) are available in published scientific literature, government documents and industry documents, or were made available to us from unpublished government and industry documents (Doyle et al, 2001). These contamination data show that *L. monocytogenes* has been detected to varying degrees in unpasteurized and pasteurized milk, high fat dairy products, soft unripened cheese (cottage cheese, cream cheese, ricotta), cooked ready-to-eat crustaceans, smoked seafood, fresh soft cheese (queso fresco), semi-soft cheese (blue, brick, monterey), soft-ripened cheese (brie, camembert, feta), deli-type salads, sandwiches, fresh-cut fruits and vegetables, and raw molluscan shellfish (Doyle et al, 2001). However, these data also show that most RTE foods do not contain detectable numbers of *L. monocytogenes*. For many RTE foods, contamination of foods with *L. monocytogenes* can be avoided - *e.g.*, through the application of current good manufacturing practices that establish controls on ingredients, listericidal and listeristatic processes, segregation of foods that have been cooked from those that have not, and sanitation. Sanitation controls include effective environmental monitoring programs designed to identify and eliminate *L. monocytogenes* in and on surfaces and areas in the plant (Doyle et al, 2001). The critical control points for food manufacturing industries will include one or more of the following:

- A packaging line is moved or modified significantly.
- Used equipment is brought from storage or another plant and installed into the process flow.
- An equipment breakdown occurs.
- Construction or major modifications are made to an area where RTE foods are processed or exposed (*e.g.*, replacing refrigeration units or floors, replacing or building walls, modifications to sewer lines).
- A new employee, unfamiliar with the operation and *L. monocytogenes* controls, has been hired to work in, or to clean equipment in, the area where RTE foods are processed or exposed.
- Personnel who handle RTE foods touch surfaces or equipment likely to be contaminated (*e.g.*, floor, trash cans) and do not change gloves or follow other required procedures before handling the food.

- Periods of heavy production make it difficult to clean the floors of holding coolers as scheduled.
- A drain backs up.
- Product is caught or hung-up on equipment. (Stagnant product in a system can be a major site of microbial growth during production.)
- Raw or under-processed foods are placed in an area designated for cooked foods.
- Frequent product changes on a packaging line cause you to change packaging film, labels, forming pockets or molds, line speeds, etc.
- Personnel are used interchangeably for packaging raw and cooked foods.
- Increased production causes you to perform wet cleaning of lines that have been taken down from production in the same room as lines that are running product.
- Heat exchangers have become compromised (e.g., with pinholes).
- Equipment parts, tubs, screens, etc. are cleaned on the floor.
- Waste bins in the RTE area are not properly maintained, cleaned and sanitized.
- Personnel handling RTE foods may come into contact with these items and then contaminate the foods and/or food contact surfaces.
- Re-circulating pumps and lines are not cleaned and sanitized.
- Indiscriminate use of high-pressure hoses in cleaning.
- Inappropriate use of footbaths in dry processing areas.

7.1 Laboratory identification of *Listeria monocytogenes*

7.1.1 Traditional method

Current methods for identification of *L. monocytogenes* rely on physiological and biochemical methods. These include Gram stain morphology, catalase, motility, beta haemolysis on blood agar and oblique illumination of colonies on blood free agar.

a. Isolation methods

Conventional methods for the isolation of *L. monocytogenes* from food, water, and soil immediately that have gained acceptance for international regulatory purposes include the United States Food and Drug Administration (FDA) method (Hitchins, 1998), the Association of Official Analytical Chemists (AOAC) official method, the ISO 11290 Standards, the United States Department of Agriculture (USDA)-Food Safety and Inspection Service (FSIS) method (FSIS, 2002.) and the French Standards (AFNOR, 2000; AFNOR, 2004). Depending on the nature of the sample, a particular method might be more suitable than others. The selective Media for the isolation of *Listeria* species include Oxford Agar, Palcam Agar, Listeria Selective Agar (Oxoid, Basingstoke, England), and Nutrient Agar Supplemented with esculine bile salt.

b. Conventional biochemical identification methods

Typical *Listeria* spp. colonies, on the above selective/differential agar plates, are then selected for further identification to the species level, using a battery of tests. The tests include the Gram-staining reaction, catalase, motility (both in a wet mount observed under phase-contrast microscopy and by inoculation into motility test media), haemolysis and carbohydrate use. The Christie-Atkins-Munch-Peterson (CAMP) test is a very useful tool to help identify the

species of a *Listeria* spp. isolates. It is used in the ISO and AOAC protocols and it is considered to be optional in the FDA and USDA-FSIS methods. The test is simple to perform and easy to read. It consists of streaking a β -haemolytic *Staphylococcus aureus* (ATCC strain 49444 or 25923, NCTC strain 7428 or 1803) and *Rhodococcus equi* (ATCC strain 6939, NCTC strain 1621) in single straight lines in parallel, on a sheep blood agar plate or a double-layered agar plate with a very thin blood agar overlay. The streaks should have enough separation to allow test and control *Listeria* strains to be streaked perpendicularly, in between the two indicator organisms, without quite touching them (separated by 1–2 mm). After incubation for 24–48 hours at 35–37°C (12–18 hours if using the thin blood agar overlay), a positive reaction consists of an enhanced zone of β -haemolysis, at the intersection of the test/control and indicator strains. *Listeria monocytogenes* is positive with the *S. aureus* streak and negative with *R. equi*, whereas the test with *L. ivanovii* gives the reverse reactions (Quinn, 1999).

7.1.2 Molecular approach for identification of *Listeria monocytogenes*

7.1.2.1 Polymerase Chain Reaction (PCR)

Enrichment and DNA extraction

A total of 25 g or 25 ml of food sample, soil, water, animal droppings, is incubated in 225 ml of *Listeria* enrichment broth (Oxoid, England) at 30 ± 1 °C for 24 and 48 h. For DNA isolation, 1 ml of suspension after 24 h and after 48 h is necessary.

Homogenate is centrifuged at $1800 \times g$ for 5 min and the supernatant is discarded. The pellet is re-suspended in 100 μ l of 0.5 % TRITON X-100 (Sigma, Germany) and the whole process is repeated. Homogenate is incubated at 95 °C for 5–10 min. 2 μ l of Proteinase K [20 mg/ml] (Promega, USA) are added to the homogenate after cooling and it is incubated at 55 °C for 2 h. Proteins are removed with a phenol-chloroform-isoamylalcohol [25:24:1] solution (Sigma, Germany). DNA is precipitated with ice-cold absolute ethanol at -70 °C for 2h, Centrifuged at $3500 \times g$ for 10 min. Pellet is dried and DNA is re-suspended in 30 μ l of sterile distilled water.

PCR amplifications

The first round used primers PRFA1 and PRFA2 (Simon et al. 1996) are directed against nucleotides 181–207 and 1462–1482 of the sequence. Each 50 μ l of the reaction mixture contains: 5 μ l target DNA, 5 μ l 10 \times PCR buffer (Gibco BRL, USA), 2mM dNTPs (Promega, USA), 50mM MgCl₂ (Gibco BRL, USA), 0.5 μ mol/l primer (Generi Biotech, Czech Republic) and 1U Taq DNA polymerase (Gibco BRL, USA), sterile distilled water added to the volume 50 μ l.

Hot start is at 94 °C for 2 min. The reaction mixtures are subjected to 35 cycles consisting of heat denaturation at 94 °C for 30 s, primer annealing at 60 °C for 30 s and DNA extension at 74 °C for 1 min. Finally, the samples are maintained at 74 °C for 5 min for the final extension of DNA. These incubation conditions are the same for second round-nested PCR, except those LIP1 and LIP2, since these primers require 45 cycles.

The second round employed primers LIP1 and LIP2 (Simon et al. 1996) directed against nucleotides 634–654 and 886–907 of first product amplified by PRFA1 and PRFA2. 2 μ l of completed first round reaction mixture are added to each reaction as target DNA. Remaining components are the same as in the first round.

Visualization of the PCR product

For detection, 10 µl of PCR reaction mixture is electrophoresed on a 2% w/v agarose gel (Gibco BRL, USA), diluted in 1 × TAE buffer (Kaufman et al. 1995), stained with ethidium bromide (Amresco, USA) in concentration 0.1 µg/ml and viewed under the ultraviolet light.

Target gene	Primer sequence (5'-3')	Amplified fragment length	Reference
prfA gene	-TCATCGACGGCAACCTCGG- -TGAGCAACGTATCCTCCAGAGT-	217 bp	Germini et al. 2009
inlA gene	-AGCCACTTAAGGCAAT- -AGTTGATGTTGTGTTAGA-	760 bp	Poyart et al., 1996.
hlyA gene	-CCTAAGACGCCAATCGAA - -AAGCGCTTGCAACTGCTC -	-	Lakićević et. al., 2010
16S rRNA Gene	- CTCCATAAAGGTGACCCT - - CAGCMGCCGCGGTAAATWC -	-	Lakićević et. al., 2010
iap gene	-GCCAGCGGCCCGGCGCGGGCCCGGCGGGG GCCGCGGCATGTCATGGAATAA- -GCTTTTCCAAGGTGTTTTT -	-	Lakićević et. al., 2010

Table 2. List of primers for detection of *Listeria monocytogenes* using polymerase chain Reaction

7.2 Practical case report: Studies on isolation of *Listeria monocytogenes* from poultry droppings in some selected farms in Okigwe, Imo State, Federal Republic of Nigeria

7.2.1 Aim and objectives of the study

The aims of this work are to:

- i. Investigate the occurrence of *Listeria monocytogenes* in poultry droppings from farms in the study area.
- ii. Study the pattern of antibiotic susceptibility of isolates of *L. monocytogenes* from the environment.

7.2.3 Study area

Poultry droppings were collected from three different farms. These farms include: David’s Poultry Farm, Harrison’s Farm, and Paul’s Poultry Farm, all in the Okigwe Local Government Area of Imo State, Nigeria.

7.2.4 Sample collection

The poultry droppings were collected using sterile universal bottles, the screw caps were carefully removed using sterile hand gloves and with the help of an applicator stick, a large

quantity of faecal droppings of poultry were collected. The samples were labeled and transported the Microbiology Laboratory, Abia State University, Uturu, Nigeria for laboratory studies.

Pre-enrichment and culture: 1 gram of faecal droppings from each sample was transferred into a test tube containing sterile and freshly prepared peptone water. This was inoculated at room temperature for 18 hours. This was to revive viable but non-culturable cells. Thereafter, a loopfull of the peptone water culture was transferred to a freshly prepared listeria Agar (Oxoid), and streaked on the surface of the solid medium. Incubation was done at 37°C for 48 hours. The pure cultures were subjected to morphologically and biochemically studies. (Nwachukwu *et.al*, 2009).

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed using the Kirby-Bauer method (Disc diffusion Technique). The discs used were manufactured by optun laboratories, Nigeria. The sensitivity discs were specifically designed and contained appropriate concentrations of different Gram positive antibiotics which include: ciprofoxacin (10µg/disc), norflaxacin (10µg/disc), gentamycin (10µg/disc), streptomycin (30µg/disc). Both cultures of different isolates of the test organism were carefully poured onto the surface of Mbellar-Hinton Agar (Previously prepared according to manufacturer’s instructions). The plates were incubated at 37°C for 48 hours. The different inhibition zone sizes were measured and recorded in millimeters (mm), and then the zone and size interpretive criteria of the National committees for Clinical Laboratory Standards (NCCLS) were used to interpret the zone sizes.

8. Results and discussions

Microbiological studies in Farm A, showed 100% frequency of occurrence of *L. Monocytogenes* from poultry droppings. In three sampling points in Farm, B, *Listeria monocytogenes* showed a frequency of 75%, 83%, and 66.6% respectively.

Farms	Numbers of Samples examined	No. of times <i>L. monocytogenes</i> was isolated (%)
Farm A	12	12 (100)
Farm A	12	12 (100)
Farm A	12	12 (100)
Farm B	12	9 (75)
Farm B	12	10 (83)
Farm B	12	8 (66.60)
Farm C	12	4 (33.3)
Farm C	12	4 (33.3)
Farm C	12	6 (66.60)

Table 3. Isolation of *L. monocytogenes* from Poultry droppings

In Farm C, *listeria monocytogenes* was isolated as frequencies of 33.3%, 33.3%, and 66% from three different locations of poultry droppings. The bacterial organism in Farm A, from poultry droppings showed a 25%, 33%, 50%, 41.7% resistance to ciprofloxacin, norfloxacin,

gentamycin, lincocin respectively. *L. monocytogenes* strains from Farm A showed 75% resistance to floxapen and Ampicillin. *L. monocytogenes* showed 83% susceptibility to tarivid (Quinolone).

In Farm 2, the highest resistance was observed against chloramphenicol (77.8%). Ampicillin (75%). The highest susceptibility rate of 77.8% was observed on Tarivid and *L. monocytogenes* isolates from Farm 2

antibiotics		Farm 1		Farm 2		Farm 3	
		(N=9)		(N=12)		(N=9)	
	µg/ disc	Ns (%)	NR(%)	Ns (%)	NR(%)	Ns (%)	NR(%)
Ciprnofloxacin	10	9(75)	3(25)	6(66.7)	3(33.3)	4(66.7)	2(33.3)
Norfloxacin	10	8(66.7)	4(33.3)	5(55.6)	4(44.4)	350.0)	2(33.3)
Gentamycin	10	6(50.0)	6(50)	3(33.3)	6(66.7)	2(33.3)	4(66.7)
Lincocin	20	7(58.3)	5(41.7)	4(44.4)	5(55.6)	5(83.3)	1(16.7)
Streptomycin	30	8(66.7)	4(33.3)	5(55.6)	4(44.4)	3(50)	3(50)
Rifampicin	20	6(50)	6(50)	3(33.3)	6(66.7)	5(83.3)	1(16.7)
Erythromycin	30	8(66.7)	4(50)	6(66.7)	3(33.3)	3(50)	3(50)
Chloram-phenicol	30	6(50)	6(50)	2(22.2)	7(77.8)	2(33.3)	4(66.7)
Ampiclox	20	6(50)	6(50)	3(33.3)	6(66.7)	2(33.3)	4(66.7)
Floxapen	20	9(75)	9(75)	6(66.7)	3(33.3)	4(66.7)	2(33.3)
Ampicillin	10	3(25)	9(75)	2(22.2)	7(77.8)	1(16.7)	5(83.3)
Tarivid	10	10(83.3)	2(16.7)	7(77.8)	2(22.2)	5(83.3)	1(16.7)

Ns; Number that remained susceptible to the antibiotic, NR; Number that was resistant to the given antibiotics

Table 4. Antibiotic Susceptibility patterns of *L. monocytogenes* isolated from different poultry droppings.

In Farm, resistance was highest in Gentamycin (66.70%), Chloramphenicol (66.70%), Ampiclox (66.70%) and Ampicillin (83.3%).

The *L. monocytogenes* strains from Farm A showed a susceptibility of 83.3%, 83.3% in Lincocin and Tarivid respectively. This observation is in accordance with the report of Walsh et.al, (2001) where *L. monocytogenes* isolates were more susceptible to Droid than floxapen.

Walsh et.al, 2004 also reported high resistance of *L. monocytogenes* to Penicillin. This drug rate of resistance to β -lactains and few quinolones could be as a result of drug abuse, and

consequent acquisition of R-plasmid by these strains of *L. monocytogenes*. From this study on poultry droppings, it is now known that drug resistant *L. monocytogenes* are available on poultry droppings. However, Poultry industries are advised to understand that poultry droppings in the farm yard remain a critical control point for the production of chickens for human consumption. In addition, the use of poultry droppings as biofertilizers should be discouraged because of the hazard of transferring *L. monocytogenes* to edible plants and finally humans.

Veterinary Surveillance, especially in the droppings of poultry must be undertaken by Public health agencies in Nigeria, Africa and the entire world.

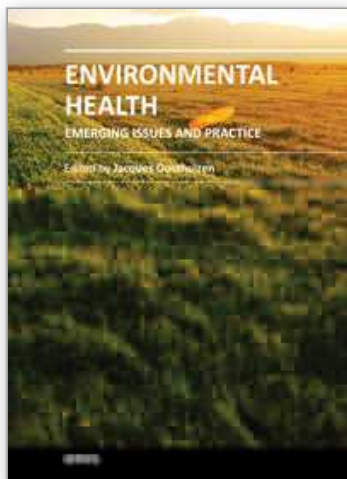
9. References

- Afnor (1997). Norme Française NF V 08-055. Microbiologie des aliments. Recherche de *Listeria monocytogenes*. Méthode de Routine. Paris, France.
- Afnor (2000). Normalisation Française XP V 08-062. Microbiologie des aliments. Méthode de dénombrement de *Listeria monocytogenes*. Méthode de Routine. Paris, France.
- Arslan S, Ozdemir F (2008) Prevalence and antimicrobial resistance of *Listeria* species in homemade white cheese. Food Control 19: 360-363
- Aureli, P. Ferrini, A.M, Hodzie, S., Wedell-Weergard, C., and Oli, B (2006). *Listeria monocytogenes* Isolated from food. International Journal of Food Microbiology, 55: 599-603.
- Bailey J., Fletcher, D., and Swaminathan B (2002). *Listeria*, *Erysipelothrix* and *Kurthia*. pp.295-314. In: manuel of Clinical Microbiology. 7th Edition Murray, R.P-(ed). American Society for Microbiology, Washington DC.
- CDC(2006). Surveillance for Foodborne-Disease Outbreaks, United States, 1998 – 2002. MMWR
- Centers for Disease Control. (1988). United States Morbidity and Mortality. Weekly Rep. 37: 377-382, 387-388.
- Dijkstra, R.G. (1978). Incidence of *L. monocytogenes* in the intestinal contents of different farm. Tijdschr. Diergeneeskde, 15: 229-231.
- Doyle ME, Mazzotta AS, Wang T, Wiseman DW, and Scott VN. (2001). Heat resistance of *Listeria monocytogenes*. Journal of Food Protection 64 (3): 410-429.
- EUAHS (2007) European Union Animal Health Strategy. Available online at <http://one-health.eu/ee/index.php/en/homepage/> (Accessed 12th July, 2011)
- Fatoki SO, Gogwana P, Ogunfowokan AO (2003) Pollution assessment in the Keiskamma River and in the impoundment downstream. Water SA 29 (3): 183-187
- Fenlon DR, Wilson J, and Donachie W. (1996). The incidence and level of *Listeria monocytogenes* contamination of food sources at primary production and initial processing. Journal of Applied Bacteriology 81 (6): 641-650. Available in Docket No. 1999N-1168, Volume 0011 bkg1 (08 of 17), Tab 134
- FSIS Risk Assessment for *Listeria monocytogenes* in Deli Meats, FDA/FSIS Risk Assessment
- Gahan, C.G.M., Hill C. (2005). Gastrointestinal phase of *Listeria monocytogenes* infection. Journal of Applied Microbiology 98, 1345-1353.

- Germini, A.; Masola, A.; Carnevali, P. and Marchelli, R. (2009). Simultaneous detection of *Escherichia coli* O175:H7, *Salmonella* spp., and *Listeria monocytogenes* by multiplex PCR. *Food Control*, 20:733-738.
- Gombas DE, Chen Y, Clavero RS, Scott VN. (2003). Survey of *Listeria monocytogenes* in ready-to-eat foods. *J Food Prot.*, 66(4):559-69
- Hitchins A.D. (1998). *Listeria monocytogenes*. In: Bacteriological Analytical Manual, US Food and Drug Administration, AOAC INTERNATIONAL, Gaithersburg, MD, USA, 10.01-10.11
- ILSI. 2005. Achieving Continuous Improvement in Reductions in Foodborne Listeriosis – A Risk Based Approach. *Journal of Food Protection* 68(9):1932-1994.
- International Commission on Microbiological Specifications for Foods (ICMSF, 2004). *Microorganisms in Foods*
- Kohne, D. E., A. G. Steigerewalt, and D. J. Brenner. (1984). Nucleic acid probe specific for members of the genus *Legionella*, p. 107-108. In: C. Thornsberry, et al. (ed.) (*Legionella*): proceedings of the 2nd international symposium. American Society for Microbiology, Washington, D.C.
- Lakićević, B. Stjepanović, A., Milijašević, M., Terzić- Vidojević, A., Golić, N. and Topisirović, L. (2010.) The Presence of *Listeria* spp. and *Listeria monocytogenes* in A Chosen Food Processing Establishment in Serbia. *Arch. Biol. Sci., Belgrade*, 62 (4), 881-887.
- Linnan, M.J., Mascola, L., Lou, X.D., Goulet, V., May, S., Salminen, C., Hird, D.W., Yonekura, M.L., Hayes, P., Weaver, R., (1988). Epidemic listeriosis associated with Mexican-style cheese. *N Engl J Med* 319, 823-828.
- Lunden JM, Miettinen MK, Autio TJ, Korkeala H (2000) Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surface after short contact times. *J Food Prot* 63:1204-1207.
- Mackintosh G, Colvin C (2003). Failure of rural schemes in South Africa to provide potable water. *Environ Geol* 44:101- 105
- Mafu A, Roy D, Goulet J, Magny P (1990) Attachment of *Listeria monocytogenes* to stainless steel, glass, polypropylene and rubber surfaces after short contact times. *J Food Prot* 53: 742-746
- Mara I. (2001). Between diarrhea disease and HIV/AIDS: debates in South Africa. Newsletter 20011021, *Health Systems Trust*, Durban, South Africa. Available via DIALOG. <http://new.hst.org.za/news/index.php/20011021/> Accessed 13 April 2009
- Maugeri TL, Carbon M, Fera MT, Irrrera GP, Gugliandolo C (2004) Distribution of 132
- Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., Tauxe, R.V. (1999). Food-related illness and death in the United States. *Emerg Infect Dis.*, 5, 607-625.
- Microbiological Specifications of Food Pathogens. Blackie Academic and Professional, New York. (1996).
- Mohammed HO, Stipetic K, McDonough PL, Gonzalez RN, Nydam DV, Atwill ER. Identification of potential on-farm sources of *Listeria monocytogenes* in herds of dairy cattle. *Am J Vet Res.*, 70(3):383-8.

- National Advisory Committee on Microbiological Criteria for Foods. (1991). *Listeria monocytogenes*. Recommendations by The National Advisory Committee on Microbiological Criteria for Foods. *International Journal of Food Microbiology* 14 (3-4): 185-246.
- Nwachukwu, N.C., Orji, F.A., and Amaike, J.I. (2009). Isolation and Characterisation of *Listeria monocytogenes* from Kunu, a locally produced beaverage marketed in different markets in Abia State of Nigeria. *Austraian Journal of Basic and Applied sciences*, 3,(4): 4432 – 4436.
- Nwachukwu, N.C., Orji, F.A., Iheukwumere, and Ekeleme, U.G. (2010). Antibiotic resistant environmental isolates of *Listeria monocytogenes* from anthropogenic lakes in Lokpa-Ukwu, Abia State of Nigeria. *Australian Journal of Basic and Applied Sciences*, 4(7): 1571-1576.
- Obi CL, Igumbor JO, Momba MNB, Samie A (2008). Interplay of factors involving chlorine dose, turbidity flow capacity and pH on microbial quality of drinking water in small treatment plants. *Water SA* 34: 565-572.
- Obi CL, Momba MNB, Samie A et al (2007) Microbiological, physico-chemical and management parameters on the efficiency of small water treatment plants in the Limpopo and Mpumalanga Provinces of South Africa. *Water SA* 33: 229-237
- Paillard D, Dubois V, Thiebaut R et al (2005) Occurrence of *Listeria* spp. in effluents of French urban wastewater treatment plants. *Appl Environ Microbiol* 71: 7562-7566
- Poyart, C., Trieu-Cuott, P., and Berche, P. (1996). The *inlA* gene required for cell invasion is conserved and specic to *L. monocytogenes*. *Microbiology*, 142, 173-180.
- Quinn P.J., Carter M.E., Markey B. & Carter G.R. (1999). *Clinical Veterinary Microbiology*. Mosby International, Edinburgh, Scotland, UK.
- Quinn P.J., Carter M.E., Markey B.K., Carter G.R. (1999). *Listeria* species. In: Quinn P.J., Carter M.E., MarkeyB.K, Carter G.R. (editors). *Clinical Veterinary Microbiology*. Mosby, Edinburgh, p. 170-174.
- Ryser, E.T. & Marth, E.H. (1991). *Listeria, Listeriosis & Food Safety*. New York, Marcel Dekker.
- Simon, C. M., Gray, D. I., Cook, N. (1996): DNA Extraction and PCR Methods for the Detection of *Listeria monocytogenes* in Cold-smoked Salmon. *Appl. Env. Microbiol.* 62: 822-824
- Slutsker L, and Schuchat A. (1999). Listeriosis in humans. In *Listeria, Listeriosis and Food Safety*, eds ET Ryser and EH Marth, 75-95. New York: Marcel Dekker, Inc. Available in Docket No. 1999N-1168, Volume 0018 bkg1 (15 of 17), Tab 389
- Swanson, K.M.J. 2005. *L. monocytogenes* Challenge Study “How To” Guidelines. *Food Safety Magazine*. June/July 2005.
- U.S. Food and Drug Administration, and U.S. Food Safety and Inspection Service. (2003). Quantitative Assessment of the Relative Risk to Public Health from Foodborne *Listeria monocytogenes* Among Selected Categories of Ready-to-Eat Foods.
- U.S. Food and Drug Administration. 2003. Chapter 10. *Listeria monocytogenes*. Detection and Enumeration of *Listeria monocytogenes* in Foods. In *Bacteriological Analytical Manual Online*.

- Vishal, S.P. (2004). Zoonotic aspects of *Listeria monocytogenes*. M.Sc. thesis submitted to Department of Food Hygiene. Swedish University of Agricultural Sciences.
- Walsh, D., Duffy, G., Sheridan, J.J., Blair S.I. and McDowell D.A. (2001). Antibiotic, *Listeria* including *Listeria monocytogenes* in: Retail foods, Journal of Applied and Environmental Microbiology, 90: 517 – 520.
- Yutaka, S., S. Naohira, D. Yohei and A. Yoshichika, 2004. *Escherichia coli* Producing CT X-m-z-betalactamase in cattle in Japan. Emerg. Infect. Dis., 10(1): 69-75.



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Environmental health practitioners worldwide are frequently presented with issues that require further investigating and acting upon so that exposed populations can be protected from ill-health consequences. These environmental factors can be broadly classified according to their relation to air, water or food contamination. However, there are also work-related, occupational health exposures that need to be considered as a subset of this dynamic academic field. This book presents a review of the current practice and emerging research in the three broadly defined domains, but also provides reference for new emerging technologies, health effects associated with particular exposures and environmental justice issues. The contributing authors themselves display a range of backgrounds and they present a developing as well as a developed world perspective. This book will assist environmental health professionals to develop best practice protocols for monitoring a range of environmental exposure scenarios.

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